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Assessment of Semiochemical Repellents for Protecting Walnut Trees From Walnut Twig Beetle (Coleoptera: Curculionidae) Attack in a Commercial Orchard Setting in California

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Abstract

The walnut twig beetle, *Pityophthorus juglandis* Blackman, the vector of thousand cankers disease (TCD), poses a significant threat to North American walnut (Juglandaceae *Juglans*) trees. Despite discovery of TCD-related tree mortality over a decade ago, management options are lacking. This study represents the culmination of several years of investigating the chemical ecology of *P. juglandis* in hopes of developing a semiochemical repellent to disrupt the beetle's host colonization and aggregation behaviors. Numbers of *P. juglandis* landing on semiochemical-treated *Juglans regia* L. trees in a commercial walnut orchard were compared based on captures on sticky traps. Two repellent combinations were tested: *R*-(+)-limonene and *trans*-conophthorin (LimeCon), and *R*-(+)-limonene, *trans*-conophthorin, and *R*-(+)-verbenone (LCV). Both repellents reduced *P. juglandis* aggregation (captures) equally; thus, we proceeded with the LimeCon combination to reduce potential treatment cost. Subsequent trials included a 2× dose (Dual) of LimeCon. Both LimeCon and Dual significantly reduced the number of *P. juglandis* caught compared with the baited control, however, only for the lower of two trap positions. Beetle landings were modeled by trap distance from repellent placement on each tree. Beetle responses to the pheromone lure were surprisingly localized and did not bring the whole tree under attack. LimeCon, LCV, and Dual treatments averaged fewer than a single beetle caught for all trap distances; however, performance of the repellents beyond 150 cm is not clear due to the localized landing response of *P. juglandis* to pheromone lures. Further testing is required to fully analyze the zone of inhibition of the LimeCon repellent.

Key words: *Pityophthorus juglandis*, thousand cankers disease, tree protection, walnut pest, walnut orchard

The insect–pathogen complex known as thousand cankers disease (TCD) currently threatens the health of North American walnut (Juglandaceae *Juglans*) trees (Tisserat et al. 2009, Kolařík et al. 2011, Seybold et al. 2016). Disease occurs when the walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), enters the phloem of a host walnut or wingnut (Juglandaceae *Pterocarya*) to feed and reproduce, thereby introducing the spores of the fungal associate *Geosmithia morbida* Kolařík, Freeland, Utley, and Tisserat (Ascomycota: Hypocreales) (Tisserat et al. 2009, Kolařík et al. 2011, Hishinuma et al. 2016, Seybold et al. 2016). TCD is characterized by localized necrosis of phloem tissue surrounding the beetle's feeding galleries. The combined activity of feeding *P. juglandis* (both adults and larvae) and the localized canker development by *G. morbida* manifest as a progressive top-down crown dieback, ultimately culminating in tree mortality (Seybold et al. 2016). Attacks are typically initiated on branches in the crown greater than 1.5–2 cm in diameter. *Pityophthorus juglandis* will also attack the main stem in later stages of host tree decline (Seybold et al. 2016). A crucial element to this complex is that *G. morbida* cannot move systemically within a host

tree. Rather, the fungus requires *P. juglandis* to vector its spores to a new host, or to new phloem tissue within the same host tree (Tisserat et al. 2009).

Both the fungal pathogen and its beetle vector are native to North America. Their range was historically restricted to portions of the southwestern United States (Arizona, New Mexico) and northern Mexico, in association with Arizona walnut, *Juglans major* (Torr.), the putative historic host (Cranshaw 2011, Seybold et al. 2016). Both organisms have established populations well beyond this historic range today. *Pityophthorus juglandis* and *G. morbida* are currently found in most of the western states, including California, Colorado, Idaho, Nevada, Oregon, Utah, and Washington, and seven eastern states, including Indiana, Maryland, North Carolina, Ohio, Pennsylvania, Tennessee, and Virginia (Cranshaw 2011; Grant et al. 2011; Tisserat et al. 2011; Fisher et al. 2013; Hadziabdic et al. 2014; Marshall 2015; Seybold et al. 2016, 2019). TCD has also been confirmed in two locations in Italy (Faccoli et al. 2016, Montecchio et al. 2016, Moricca et al. 2019). The beetle and pathogen have likely been moved around through the movement of infested wood (Newton and Fowler 2009, Jacobi et al. 2012).

Establishment of *P. juglandis* and *G. morbida* well beyond their historic native range has significant economic and ecological implications. In California, English walnut, *Juglans regia* L., orchards represent a $1.4 billion a year industry (Walnut Board 2019). *Juglans regia*, a non-native species, is a known host of *P. juglandis* (Leslie et al. 2009, Seybold et al. 2016, Hefty et al. 2018) and is susceptible to the fungal pathogen as well (Utley et al. 2013, Yaghmour et al. 2014). Eastern black walnut, *J. nigra* L., is a particularly valuable timber species, with both major plantings in Illinois, Indiana, Kentucky, Missouri, and neighboring states and forests across a broad area in the eastern United States. Estimates indicate a value of greater than $500 billion in standing black walnut timber in eastern forests and plantations (Newton and Fowler 2009). Walnuts have also been planted as landscape and street trees in towns and cities across the United States (Seybold et al 2019). Removal of large numbers of landscape trees as a result of insect pest outbreaks can be costly to city and county governments (Kovacs et al. 2010, Bigsby et al. 2014).

Native North American walnut trees are an important riparian forest species that provide a mast resource for wildlife (Barnes et al. 1998). The northern California black walnut, *Juglans hindsii* (Jeps.) Rehder, and southern California black walnut, *Juglans californica* S. Wats., have limited native distributions in northern California/Oregon and southern California, respectively (Griffin and Critchfield 1976, Keeley 1990, Callahan 2008). There is both economic and ecological imperative to manage the spread of this devastating insect–pathogen complex and to mitigate the impacts in those areas where *P. juglandis* and TCD have established.

Despite this imperative, management options for TCD are lacking. Focus has primarily been on detection of the beetle and pathogen, monitoring of *P. juglandis*, and placement of quarantine restrictions on walnut wood and nursery stock (Newton and Fowler 2009; Seybold et al. 2016, 2019). Effective methods for steam heat sanitation (Mayfield et al. 2014) and methyl bromide fumigation (Audley et al. 2017, Seabright et al. 2019) of walnut logs and slabs have been developed. However, current recommendations for infested trees include removal and sanitation. Chemical control methods and recommendations for *P. juglandis* and/or *G. morbida* on live trees are currently lacking in the literature. According to University of California Integrated Pest Management Guidelines for walnut, chemical control strategies are not recommended to manage *P. juglandis* populations in orchards (Grant et al. 2017). Systemic insecticides and fungicides have been proposed as a viable strategy. Dal Maso et al. (2019) reported abamectin (insecticide) with prochloraz and tetraconazole (fungicides) as being effective in laboratory assays against both organisms. Preliminary results from trials of emamectin benzoate (insecticide) and thiabendizole (fungicide) in *J. regia* in California yielded mixed results (Munson et al. 2016).

The cryptic nature of *P. juglandis* further complicates the use of insecticides. To achieve effective surface sprays, insecticide formulations must be administered to the bark surface prior to beetle colonization (Seybold et al. 2008, Fettig and Hilszczański 2015), but not so far in advance for the formulation to degrade or wash off prior to beetle arrival (Seybold et al. 2008). This is particularly problematic as *P. juglandis* are known to be active at temperatures at or above 18–19°C, and beetle activity has been observed in every month of the year in California, although *P. juglandis* typically emerge in February–March and have two main flight periods, May–June and September–October (Chen and Seybold 2014). This suggests that trees need to be protected from *P. juglandis* colonization for most of the year, perhaps requiring multiple insecticide treatments. Beyond logistical and economic constraints, insecticides intended to target twig and bark beetle pests may also kill nonpest, minor pest, and/or beneficial insects (Silverstein 1981, Fettig et al. 2013, Seybold et al. 2018). Trunk sprays are also likely to cause some level of environmental contamination beyond direct arthropod mortality as well. These issues often limit insecticide applications to high-value individual trees or groups of trees in bark beetle pest systems (Wood 1980, Silverstein 1981, Wood et al. 1985, Borden 1997, Seybold et al. 2018).

We argue that a semiochemical repellent approach may be a viable alternative for protecting walnut trees from *P. juglandis* and TCD. Evidence suggests *P. juglandis* are capable of in-flight discrimination between host and nonhost trees (Homicz et al. 2020, Audley et al. 2020a) and among *Juglans* spp. (Hishinuma 2017, Lona et al. 2020). It appears likely *P. juglandis* utilizes host and nonhost cues to help guide this discrimination. Volatile compounds potentially repellent or interruptive to *P. juglandis* host selection and aggregation behaviors include *R*-(+)-limonene (Blood et al. 2018, Audley et al. 2020b), *trans*-conophthorin, and *R*-(+)-verbenone (Audley et al. 2020c). All three compounds successfully reduced the number of *P. juglandis* caught in trapping assays when presented individually and in combinations. Reducing the number of *P. juglandis* caught in baited traps is certainly indicative of repellent/avoidance behaviors, however, an effective repellent tool must translate into effective tree protection.

The objective of this study was to measure the efficacy of two potential repellent combinations in protecting individual walnut trees in a commercial orchard setting from colonization by *P. juglandis*. We also focused on elucidating the effective protected radius (zone of inhibition) provided by each repellent. The ultimate intent of this work was to develop an effective management option for protecting walnut trees from the impacts of TCD.

Materials and Methods

Study Site and Tree Selection

This study was conducted in an approximately 300 m × 140 m, rectangular section of a commercial English walnut orchard (*J. regia* on *J. hindsii* rootstock) located directly adjacent to the western edge of the Wolfskill Experimental Orchards property (property of the University of California, on which several *P. juglandis*-related studies

have taken place; see Chen and Seybold 2014; Hishinuma 2017; Audley et al. 2020b,c; Lona et al. 2020) and south of Putah Creek in Winters, CA. Trees were Hartley cultivars planted in 1983 (note that a few trees had been removed and replaced since the first planting). Only trees from the original planting were used in the study (based on overall size and diameter at breast height). This site was selected based on the property's proximity to a known, prolific population of *P. juglandis* on the adjacent Wolfskill property.

To assess the population distribution of *P. juglandis* throughout the orchard, we first established a grid of four-unit, Lindgren funnel traps baited with 15-ml polyethylene bottles filled with 3-methyl-2-buten-1-ol (MBO; Sigma–Aldrich product #162353, 5 mg/d release rate at 30°C), the primary component of the male-produced aggregation pheromone (Seybold et al. 2013, Seybold et al. 2015). Prior to Trial 1, nine traps were placed in the orchard in an evenly spaced 3 × 3 trapping array. The array began in the north-east corner of the block, two trees from the eastern edge and two rows from the southern edge of the block. Traps were spaced ~80 m apart in a 3 × 3, square grid. The initial trapping array was established on 4 April 2018. Trap samples were collected every 1–3 wk and transported to the lab for sorting. Upon the start of Trial 1 (14 June 2018), the monitoring trapping array was reduced from nine traps to three, retaining the middle row of traps which was roughly in the middle of the trees included in the study. Weekly monitoring of the three traps continued throughout the duration of Trial 1, continued throughout the summer between Trials 1 and 2, and through the conclusion of Trial 2 in October 2018. The following spring, the nine-trap array was established in roughly the same positions on 8 April 2019 and monitored until 6 May 2019 (to confirm a similar distribution of *P. juglandis* activity). At this time, the trapping array was again reduced to the middle row of three traps and monitored weekly through the conclusion of Trial 3 on 29 May 2019.

Based on the homogenous distribution of *P. juglandis* recovered from our monitoring traps (*see* results), we used a randomized design with repeated measures for all three trials. Trees selected for the study were at least 15 m away from the nearest selected tree, one tree away from an edge, and a buffer tree was included between any two trees assigned to a treatment to reduce potential cross-treatment semiochemical influence and to allow space for measuring for the zone of inhibition around treated trees. In Trial 1, 60 trees (15 trees per treatment) were selected and randomly assigned to one of four treatment categories. In Trial 2, the 15 trees assigned to the negative controls were retained and 45 new trees were selected and randomly assigned to the remaining three treatments. Most of the newly selected trees for Trial 2 served as buffer trees during the first trial. In Trial 3, we selected 60 new trees, beginning one row to the north of the beginning row of the previous two trials (i.e., the buffer rows from the first two trials).

All trees, except those assigned to the negative control group, were baited with the *P. juglandis* pheromone. The 15-ml MBO lures were placed at a height of 1.75 m above the soil line. The repellent semiochemical release devices were placed adjacent to the lure except in the case of the Dual treatment (see below). All semiochemical release devices were secured to the tree on wire wrapped around thumbtacks pushed into the bark. Semiochemical placement was standardized to the north face of each tree bole.

Clear acetate sheets (two 21.6 × 28 cm sheets taped together) covered in StickEm Special (Seabright Laboratories, Emeryville, CA) were deployed to capture *P. juglandis* landing on the trees in our study. Two sheets were placed on each tree. One sheet was placed on a lower scaffolding branch, or in many cases the main stem, ~2 m above the soil line on a randomly selected side (cardinal direction) of each tree. The second sheet was then placed around a higher branch at ~4 m in height, on the opposite side of the tree from the lower sheet. See Supp Mater (online only) for a diagram of the study set-up. Sticky traps were secured using push pins placed into corks glued to the bark surface with Elmer's WoodGlue (Elmer's Products Inc., Westerville, OH).

Landings on Treated Trees

Trees ($n = 60$ trees, 15/treatment) were randomly assigned to four treatments: 1) positive control; 15-ml polyethylene bottle with MBO only (henceforth, 'MBO lure'); 2) MBO lure plus 15-ml *R*-(+)-limonene (Sigma–Aldrich product #183164-500ML, lot #MKBN6863V, 97% chemical purity) in polyethylene bottle (700 mg/d release rate at 30°C) plus 250-µl *trans*-conophthorin (50% (+) and 50% (−), 95% *trans* and 5% *cis*, ChemTica, Heredia, Costa Rica) in a 400-µl polyethylene tube (3 mg/d release rate at 30°C; henceforth referred to as 'LimeCon'); 3) MBO lure plus LimeCon plus 70 g of SPLAT Verb (ISCA Technologies Inc., Riverside, CA; 7 g *R*-(+)-verbenone per 10 g proprietary carrier formulation, 28.6 mg/d release rate at 30°C; Fettig et al. 2015; henceforth, 'LCV'); and 4) negative control; no semiochemicals placed on the tree. Trees were randomly assigned to a treatment as previously described. Trial 1 was conducted from 14 June to 18 July 2018 (Fig. 1).

Analyses from Trial 1 (see Results) indicated LCV treatment did not perform better than the LimeCon treatment and therefore the LCV treatment was removed. Treatments for Trial 2 were the same as Trial 1 except a Dual treatment replaced LCV. The Dual treatment was a double dose of the LimeCon treatment, in which one set of repellents was placed at 1.5 m and the second placed at ~4 m in height on the tree. The MBO lure placement remained at 1.75 m in height. Trial 2 was conducted from 13 September to 11 October 2018 (Fig. 1). Trial 3 was a complete replicate of Trial 2 and was conducted from 6 to 29 May 2019 (Fig. 1).

Zone of Inhibition

We were also interested in determining the distance beyond a single tree where the repellent may be effective. During Trial 2, a subset of the trees ($n = 20$ trees, 5/treatment) received two 5-m-tall poles that were placed around the selected trees (one pole at 5-m distance from the stem, randomly assigned to either the east or west side of the tree, and the second pole on the opposite side at 10 m). Two smaller sticky traps (21.6 × 28 cm sheets) were wrapped around the poles, one at 2 m and the second at 5 m in height on the poles. The sheets were fastened to the poles on two corks glued onto the pole. A single MBO bubble cap lure (Contech Enterprises Inc., Product #300000968, Lot #14174, Delta, BC, Canada; released at 1.2 mg/d at 25°C) was pinned to the top cork beneath each trap. Poles were placed over 2-m-long rebar hammered vertically into the ground. Based on the trapping results from the sheets placed onto poles from Trial 2 (see Results), we reduced the pole height to 3 m, and only placed a single sheet per pole at 3 m in height during Trial 3. We also only placed a single pole at 5 m from the stem (again randomly assigned to the east or west side of each tree); however, we doubled the number of replicates to 10 ($n = 40$).

Early observations from Trial 1 suggested that fewer *P. juglandis* were caught on sticky traps placed on higher branches, even within the positive control. Thus, we wanted to quantify the effect of distance from each trap to the semiochemicals on each tree. Distances were measured from the nearest semiochemical to the mid-point of each trap using a tape measure. Trap distances on the negative control trees were measured to the same area of the tree where the lure

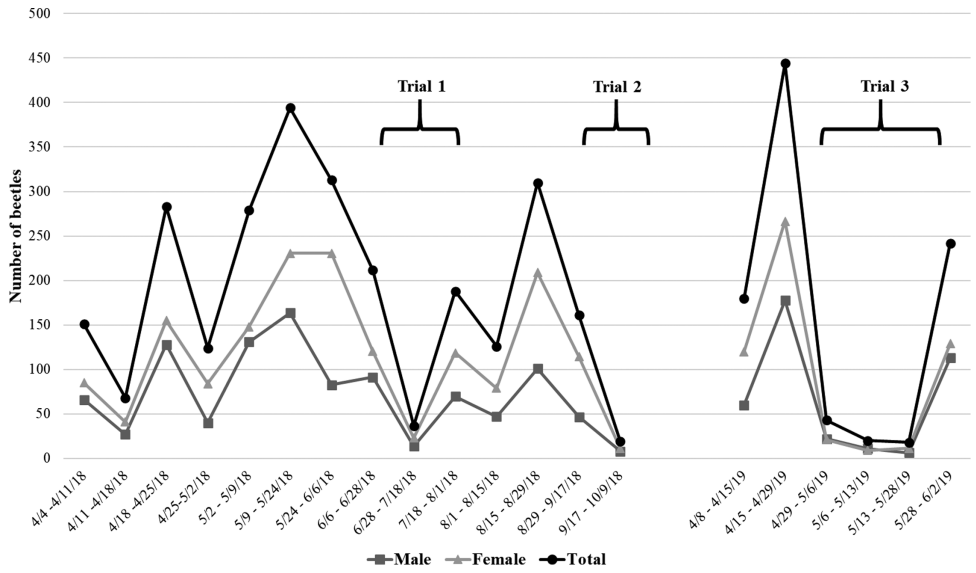

Fig. 1. *Pityophthorus juglandis* flight activity based on captures in 4-unit Lindgren funnel traps baited with a 15-ml polyethylene bottle with 3-methyl-2-buten-1-ol. A nine-trap grid (3 × 3) was used in April–May 2018 and April 2019 and was subsequently reduced to the middle three traps for the duration of 2018 and 2019. The flight curves represent the total trap catch per week. Brackets indicate when each trial was conducted in relation to the flight monitoring trap catch.

would be placed on corresponding positive control trees. This metric allowed us to further analyze the spatial efficacy of the repellents.

Data Handling

At the conclusion of each trial, sticky traps were removed and placed into 32 × 32 × 3.2 cm boxes for transport to the laboratory. Each sheet was then examined under a heat lamp to assist with removing insects from the traps. All coleopterans were removed from each sheet to ensure no *P. juglandis* were missed. Removed insects were then placed into vials containing xylene for at least 3 d to dissolve the remaining adhesive. Each sample was then poured onto tissue paper and placed outside of the laboratory for at least 1 h for the xylene to evaporate. All dried samples were then examined under a dissecting scope (40× magnification). All *P. juglandis* were tallied by sex. Following identifications, all samples were placed into vials containing 100% ethanol for preservation.

Statistical Analyses

Generalized linear mixed effects regressions were implemented to identify significant covariates following a similar approach to that of Komonene et al. (2011). We fit each data set to a poisson distribution; however, these data were overdispersed in all cases based on the dispersiontest() function in the pscl package. Therefore, analyses were conducted using the negative binomial distribution with a log link(glm.nb). We treated each tree as a random effect in the models to account for the repeated measure (traps at two locations) on each tree. Model selections were informed using Akaike information criterion values and likelihood ratio tests (Zuur et al. 2009). When applicable, pairwise, multiple means comparisons among treatments were made using post hoc pairwise, least squares means (lsmeans) tests with the Bonferroni correction method using the emmeans package (Lenth et al. 2019).

The initial *P. juglandis* flight surveys were analyzed for differences in weekly trap catch across the nine traps. We used a mixed effects model again to account for the repeated measures for each trap and treated the collection week as a random factor. Pairwise, multiple means comparisons were made using the least squares means tests with the Bonferroni correction. The spring trap catches in both 2018 and 2019 were analyzed to justify the use of a randomized design discussed during tree selection (see above).

For the sticky traps placed on the trees, the total number of *P. juglandis* males and females were compared (sexes analyzed independently). All models initially began with treatment, trap position (low branch vs. high branch), and branch direction (N, S, E, or W) as explanatory factors (fixed effects) with each tree as random effects variables. Interactions among the factors were also considered. A similar approach was implemented for traps placed on poles in Trials 2 and 3. Models for Trial 2 considered treatment, distance from the tree (5 m vs 10 m), height on the pole (2 m vs 5 m), and direction of the pole relative to the tree (E vs W). For Trial 3, only treatment and direction of the pole were evaluated as distance from the tree and height on the pole were constant (5 m distance and 2 m height). Covariates in these models were treated as categorical variables. During the trials, 14 sticky traps were lost (likely blew away) and were omitted from analyses.

Finally, *P. juglandis* responses were modeled against the trap distance (continuous numerical variable) from the nearest semiochemicals on each tree. Response was analyzed for total numbers (males + females) of beetles caught in response to the positive control (n = 83), in response to all semiochemical repellent treatments (LimeCon, n = 85; LCV, n = 30; Dual, n = 59), and in response to all negative control treatments (n = 91). Data were pooled across trials as the trial variable was not significant as either a main effect or as a random variable. Negative binomial GLM regressions were fit to each of the three groups and predicted values were generated with a 95% confidence interval.

Analyses were conducted using the lme4, emmeans, MASS, and ggplot2 packages with R Statistical Software (version 3.4.4) via RStudio (version 1.2.1335) statistical software (R Core Team 2019).

Results

Initial Flight Survey

In spring 2018, survey traps caught an average of 9.3 ± 4.4 (trap 7) to 50.4 ± 11.8 (trap 9) *P. juglandis* (mean ± SEM) per week. Wald's test indicated that trap was a significant factor (χ^2 = 72.77,

df = 8, $P < 0.01$) and pairwise, multiple means comparisons indicated that trap 9 differed significantly from six of the other eight traps (all $P \leq 0.01$) and trap 3 differed from four other traps (all $P \leq 0.03$). The greatest number of *P. juglandis* caught occurred in the three traps along the third (northern most) trapping row. As a result of the elevated number of *P. juglandis* caught in traps along this row, we selected rows of trees around the first two rows of funnel traps (average weekly, per-trap catch ranged from 9.3 to 19.6 and did not differ among these six traps). Rows selected for inclusion in the study, though populated by slightly fewer *P. juglandis* than the northern-most rows, had a fairly uniform distribution of beetles allowed for a completely randomized design. We felt that an average per-week funnel trap catch of 16 beetles was indicative of sufficient potential colonization pressure for the study.

A similar distribution of *P. juglandis* was observed again from the 2019 spring funnel trapping survey. Weekly mean number of *P. juglandis* caught per trap per week ranged from 16.7 ± 0.9 (trap 4) to 64 ± 24.9 (trap 9). Traps were a significant factor in the model ($\chi^2 = 37.04$, df = 8, $P < 0.01$). Fewer differences in trap catches existed in the 2019 trap catch data, with the two traps catching the fewest *P. juglandis*, traps 4 and 7, differing from traps 2, 3, and 9 (all $P \leq 0.04$). Despite these two traps catching fewer beetles, we felt the distribution of beetles justified a randomized design.

Landings on Treated Trees

In total, 150 *P. juglandis* (54 males, 96 females) were recovered from 117 sticky traps during Trial 1. Treatment and treatment × trap position were significant variables in explaining the observed beetle responses. Cardinal direction of trap position was not significant in the model, indicating that *P. juglandis* did not discriminate which side of a given tree to land on. Post hoc pairwise, multiple means comparisons indicated that significantly more *P. juglandis* landed on the positive control, low branch placement traps for both sexes (males: all *z*-ratios ≤ −3.26, all $P \leq 0.03$; females: all *z*-ratios ≤ −4.45, all $P < 0.01$; Fig. 2). The positive control, low traps averaged 2.6 ± 0.8 male and 5.3 ± 1.7 female *P. juglandis*, whereas all other traps averaged fewer than 0.5 *P. juglandis* (Fig. 2). No difference existed in the mean number of beetles caught on LimeCon- versus LCV-treated trees at either trap position for either sex of *P. juglandis*.

During Trial 2, a total of 2,674 *P. juglandis* were recovered from sticky traps; however, 1,233 were recovered from a single low position trap on a positive control tree. This trap was removed as an outlier leaving 1,441 *P. juglandis* (492 males, 949 females) from 113 sticky traps placed on trees for the analysis. As with the first trial, the model indicated treatment × trap position best explained the data. Traps at the low position on positive control trees caught significantly more *P. juglandis* than all other traps for both sexes (all $P < 0.01$; Fig. 3). An average of 4.1 ± 1.2 male and 8.8 ± 2.8 female *P. juglandis* landed on the lower traps on positive control trees, whereas all other traps averaged fewer than 0.5 *P. juglandis*. No differences existed among LimeCon, Dual, and the negative control for either sex (Fig. 3).

In total, 283 *P. juglandis* (90 males, 193 females) were recovered from 118 sticky traps during Trial 3. Once again, the selected model indicated treatment × trap position best explained the data. As with the previous two trials, low trap position on positive control trees caught significantly more *P. juglandis* than other treatments and trap position combinations for male and female beetles (all $P \leq 0.01$, Fig. 4). The average number of *P. juglandis* recovered from positive control low traps was 5.3 ± 1.3 and 11.4 ± 3.4 for males and females, respectively. All other traps averaged fewer than a single *P. juglandis* except for the Dual low trap which caught 1.3 ± 0.5 beetles (Fig. 4).

Zone of Inhibition

In total, 3,087 *P. juglandis* (869 males, 2,216 females) were recovered from the 79 sticky traps placed on poles near treated trees during Trial 2. Trap height on the poles was the only significant factor in the model for both males and females. No treatment effect was observed at either 10 or 5 m pole placements. More male and female *P juglandis* were recovered from baited sticky traps at a height of 2 m than at 5 m

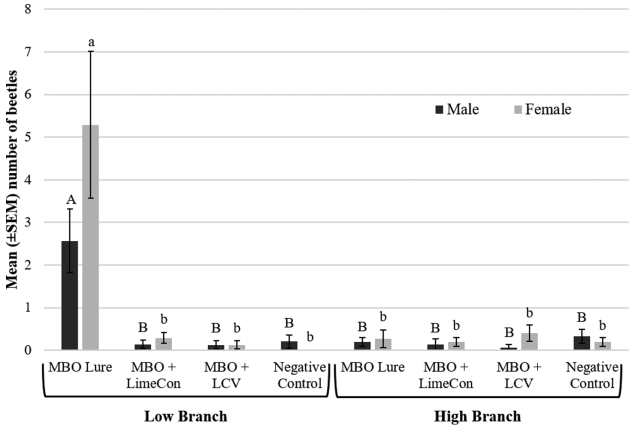

Fig. 2. Behavioral responses of *Pityophthorus juglandis* to *Juglans regia* baited with 3-methyl-2-buten-1-ol (MBO) lures, MBO and *R*-(+)-limonene + *trans*-conophthorin (LimeCon), MBO and *R*-(+)-limonene + *trans*-conophthorin + *R*-(+)-verbenone (LCV), and no semiochemicals (negative control) in Trial 1, 13 June–18 July 2018 in Winters, CA. Mean number (±SEM) of male and female *P. juglandis* recovered are depicted. Analyses were conducted by using negative binomial generalized linear mixed effects regression models in R. Multiple means comparisons were made using *posthoc* pairwise, least squares means tests with the Bonferroni correction method. Different letters indicate significant differences between means ($\alpha = 0.05$) with male and female comparisons made separately.

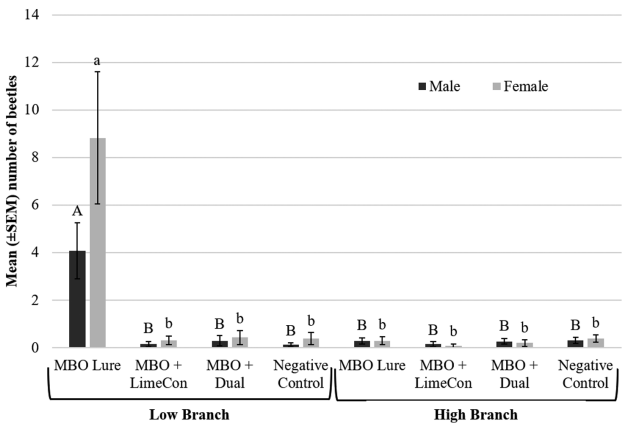

Fig. 3. Behavioral responses of *Pityophthorus juglandis* to *Juglans regia* baited with 3-methyl-2-buten-1-ol (MBO) lures, MBO and *R*-(+)-limonene + *trans*-conophthorin (LimeCon), MBO and a double dose of *R*-(+)-limonene + *trans*-conophthorin (Dual), and no semiochemicals (negative control) in Trial 2, 13 September–11 October 2018 in Winters, CA. Mean number (±SEM) of male and female *P. juglandis* recovered from each trap are depicted. Analyses were conducted by using negative binomial generalized linear mixed effects regression models in R. Multiple means comparisons were made using *posthoc* pairwise, least squares means tests with the Bonferroni correction method. Different letters indicate significant differences between means ($\alpha = 0.05$) with male and female comparisons made separately.

regardless of distance from the treated trees (z-ratio = 3.16, P = 0.002 for males; z-ratio = 2.3, P = 0.02 for females). Traps at 2 m averaged 11.1 ± 1.2 males and 25.2 ± 2.2 females and traps at 5 m averaged 6.5 ± 1.0 males and 17.3 ± 2.3 females.

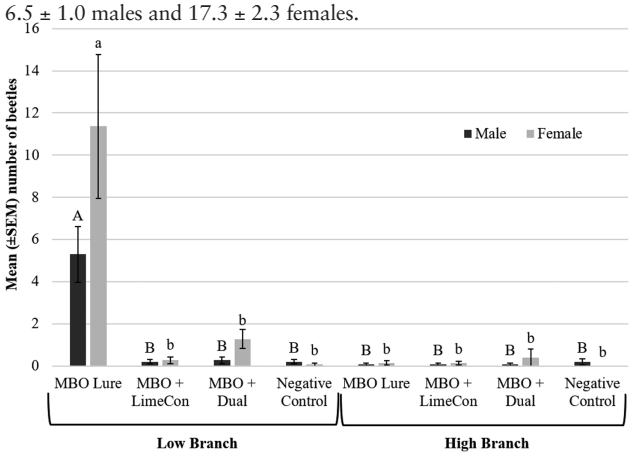

Fig. 4. Behavioral responses of *Pityophthorus juglandis* to *Juglans regia* baited with 3-methyl-2-buten-1-ol (MBO) lures, MBO and *R*-(+)-limonene + *trans*-conophthorin (LimeCon), MBO and *R*-(+)-limonene + *trans*-conophthorin + *R*-(+)-verbenone (LCV), and no semiochemicals (negative control) in Trial 3, 2–30 May 2019 in Winters, CA. Mean number (±SEM) of male and female *P. juglandis* recovered from each trap are depicted. Analyses were conducted by using negative binomial generalized linear mixed effects regression models in R. Multiple means comparisons were made using post hoc pairwise, least squares means tests with the Bonferroni correction method. Different letters indicate significant differences between means (α = 0.05) with male and female comparisons made separately.

During Trial 3, a total of 2,499 *P. juglandis* (655 males, 1,844 females) were collected on 40 baited sticky sheets placed on poles at 5 m from treated trees. The best models to explain these data indicated that the interaction term treatment × direction (east or west) was significant for both males and females. Lsmeans pairwise comparisons indicated that the only significant difference in means was between the positive control east placement (10.9 ± 4.1), and the positive control west placement (7.3 ± 1.7; z-ratio = 3.5, P = 0.02) for male beetles. When the direction term was ignored, there was no observed treatment effects for either sex.

Trap distances from semiochemicals for sticky traps placed onto trees across the three trials ranged from 17.8 to 419.1 cm and were fairly normally distributed. Trap distances were significantly farther away from semiochemicals at the higher branch position (mean = 186.8 ± 4.2 cm) than for the lower branch position (mean = 84.6 ± 2.5 cm; χ^2 = 211.87, df = 1, P < 0.001). Trap distances within trap position did not differ among treatments.

Pityophthorus juglandis captures on positive control trees were negatively correlated with trap distance from the MBO lure (Fig. 5A). Only five individuals were caught on traps placed >200 cm from the lure. The predicted values curve indicated that the number of expected beetles caught declines sharply between 0 and 150 cm away from the pheromone lure and the value approaches zero as distance approached 200 cm. The farthest trap distance from which a beetle was recovered was 218 cm.

Beetle responses to the three repellent treatments (LimeCon, LCV, and Dual) were modeled without regard to the different treatments as the previous models indicated no significant differences in

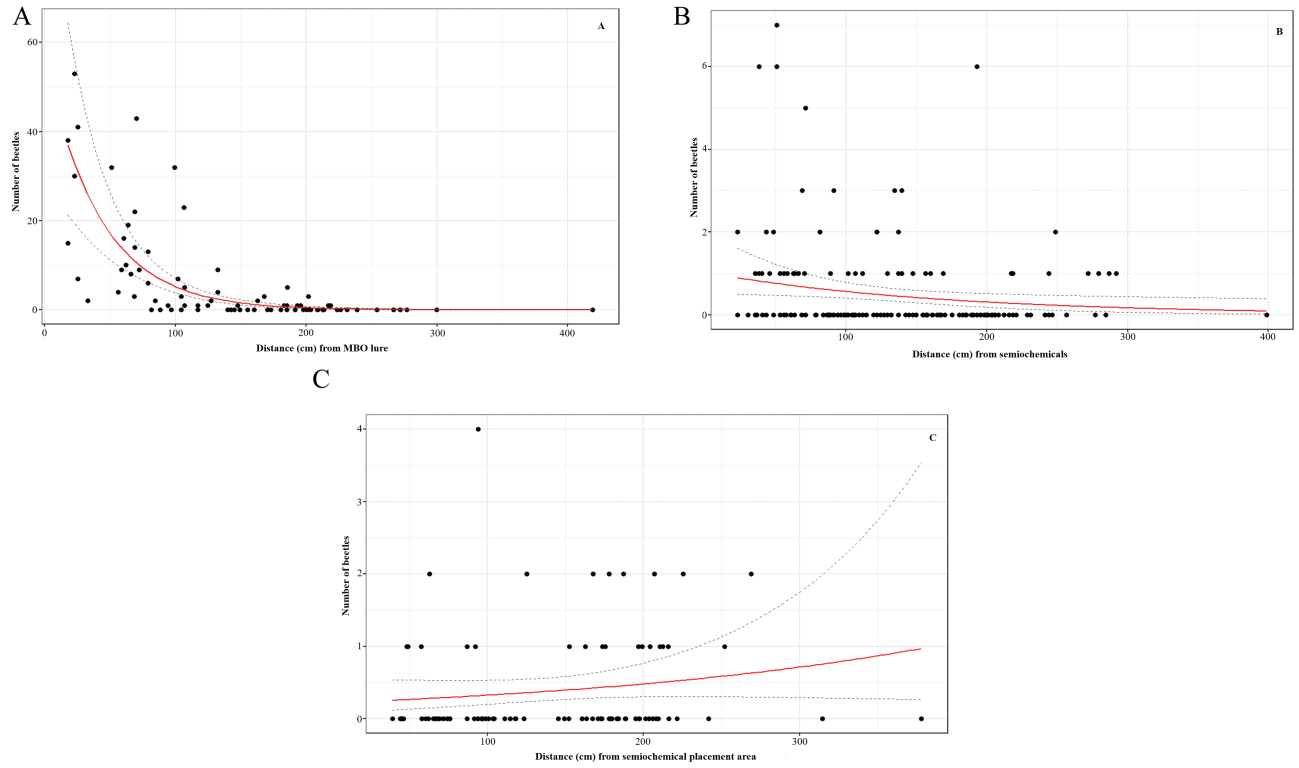

Fig. 5. Scatter plots of the total *Pityophthorus juglandis* recoveries pooled across the three trials and plotted against the distance (cm) each sticky trap was placed from semiochemicals on each tree. Trees baited with 3-methyl-2-buten-1-ol (MBO) were analyzed together (A), the three semiochemical repellent treatments, MBO and *R*-(+)-limonene + *trans*-conophthorin (LimeCon), MBO and *R*-(+)-limonene + *trans*-conophthorin + *R*-(+)-verbenone (LCV), and MBO and a double dose of *R*-(+)-limonene + *trans*-conophthorin (dual) were analyzed together (B), and all negative control trees were analyzed together (C). Each data set was fit to a negative binomial generalized linear model. This model was used to predict the number of *P. juglandis* caught for a given trap distance. Predictions are represented by the solid curves with a 95% confidence interval (dashed curves).

mean beetle trap catch. Beetle response was negatively correlated with distance from the repellents (Fig. 5B), though the curve declines only slightly and appears relatively flat. The predicted values curve approached zero between 300 and 400 cm from the repellents. Even at the closest trap distances, the predicted number of *P. juglandis* is slightly below 1 beetle. In total, nine *P. juglandis* were recovered from eight traps beyond 200 cm.

Predicted values based on the model fit to the negative control traps indicated a slight positive correlation between the number of *P. juglandis* caught and trap distance from the semiochemical placement area (Fig. 5C). Predicted *P. juglandis* catch approaches 1 beetle between 350 and 400 cm, though the confidence interval widens substantially around the predicted curve beyond 250 cm. Observed numbers of *P. juglandis* on the traps ranged from 0 to 4 *P. juglandis* and a total of 36 *P. juglandis* were recovered across the three trials. Eleven of the 36 *P. juglandis* were recovered from traps placed >200 cm from the semiochemical placement area. Interestingly, more *P. juglandis* were recovered from high traps (22) than from low traps (14).

Discussion

Results of the three trials presented provide compelling evidence that the semiochemical repellents tested reduce the number of *P. juglandis* landing on treated trees, thus confirming our first hypothesis. The addition of *R*-(+)-verbenone (LCV) did not confer any additional reduction in the number of *P. juglandis* recovered from sticky traps (Fig. 2) and was thus eliminated from subsequent trials. We also expected fewer beetles to be recovered from sticky traps on trees treated with the double dose of LimeCom (= Dual); however, the Dual treatment did not perform better than a single dose of LimeCon. A particularly interesting observation was the consistently low number of *P. juglandis* recovered from the high traps on positive control trees (Figs. 2–4). It appears that our results were limited by a localized response to the aggregation pheromone lure.

We do acknowledge that we missed *P. juglandis* peak flight activity per our monitoring trapping grid in each of the three trials (Fig. 1). This may have impacted our results; however, we believe we still captured sufficient activity to demonstrate the efficacy of the repellent at a local scale on individual trees. We missed the peak flight activity during Trial 1 for logistical reasons. Trials 2 and 3 were started within a week of recording what was ultimately the peak flight activity in both seasons. At the time of implementation, we had no apparent indications that activity would decline so rapidly. The sharp decline in flight activity during Trials 1 and 3 are best explained by weather. Several days during Trial 1 were very windy, thus impeding *P. juglandis* flight. The trough experienced in Trial 3 is explained by unusually late, cool rainstorms, during the spring of 2019.

Of note, the MBO lure did not appear to bring the entire tree under attack by *P. juglandis*. This is evident in the concentrated nature of *P. juglandis* captures within 150 cm of the lure (Fig. 5A). One potential explanation is that *P. juglandis* may aggregate in greater population densities within a given section of a host tree before secondary and/or anti-aggregation signals elicit dispersal to unoccupied phloem space than we achieved via baiting during our study. Bark beetles are known to regulate their population densities on host trees to prevent over exploitation of phloem resource that would negatively affect progeny (Raffa et al. 2016). This is often achieved through secondary pheromonal compounds that become repellent, eliciting avoidance or dispersal behaviors above a concentration threshold (Raffa et al. 1993, 2016). Without this signal, *P. juglandis* may be drawn to within close proximity of the lure rather than spreading out throughout a given tree. Lona et al. (2020) reported observing most *P. juglandis* attack (entrance) holes within 19 cm of the MBO lure when placed on walnut branches. A propensity to aggregate close to the pheromone source would help explain the paucity of *P. juglandis* recovered from traps beyond 150 cm (i.e., high trap positions).

Another possible explanation for the lack of beetles landing higher in the crowns may be that *P. juglandis* were not flying higher in the canopy in this orchard. Conventional wisdom is that *P. juglandis* initiate colonization of a host tree in the upper crown (Tisserat et al. 2009, Seybold et al. 2016); however, we captured more beetles at 2 m than at 5 m on baited traps (traps on poles) during this study. It is possible that beetles would opt for flying well below the canopy in an orchard setting where there is little to no understory growth (that might interfere with dispersal) versus the more dense canopy.

Our results clearly show that all three repellents tested reduced the number of *P. juglandis* landing on the low position traps compared to positive control traps (Figs. 2–4). This is also reflected in the predicted values curve for the pooled repellents (Fig. 5B). Our modeling predicts the recovery of only a single beetle at trap distances up to 100 cm, and fewer than 1 beetle between 100 and 200 cm from the repellent. Observations of *P. juglandis* on traps placed on trees of >200 cm from the semiochemicals were consistently low (a total of 9 beetles) on trees treated with the repellents, and these trap catches are consistent with the numbers observed from the negative controls (Figs. 2–4 and 5C). It cannot be determined from these data if the repellent semiochemicals were affecting *P. juglandis* landing behavior beyond 150 cm away.

The relatively localized repellent effect is further corroborated from observations of baited sticky traps placed on poles around treated trees. We observed no significant reduction in the number of *P. juglandis* caught even when poles were placed 5 m from trees. It was interesting that even Dual was not enough to overcome the 1.2 mg/d MBO attractant at 5 m. Furthermore, we saw no evidence to suggest the repellents were inhibiting *P. juglandis* attraction beyond the individual tree. Rather, the results of this study would indicate the necessity to treat at least each tree in a stand or orchard. In fact, it appears likely from these data that multiple application points per tree may be required, especially for trees with large crowns. This may prove to be prohibitively costly, as the estimated cost of the most effective repellent (LimeCon) is $12.81 a unit ($2.22 for 15 ml of *R*-(+)-limonene and $10.95 for each *trans*-conophthorin tube). However, further reductions in cost may be possible. For instance, transgenic microbial pheromone 'breweries' that harness genetically modified microbes could be used to produce chemically pure stereoisomers of semiochemicals at vastly reduced costs (Gillette and Fettig 2020). It should be noted that the scale of this study was focused at the individual tree level. It remains to be seen if there is a concentration of repellent compounds at the stand or orchard level whereby beetles would be deterred from the area altogether; however, based on these results, that seems unlikely.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

Fig. 1. Diagram of the experimental set-up in the commercial walnut orchard. Sticky traps are represented by gray rectangles on trees and poles (black lines). White rectangles on trees represent 3-methyl-2-buten-1-ol (MBO) lures and white triangles represent semiochemical repellents. Letters indicate aspects of the

set-up that changed across the three trials. (A) indicates the location on the trees where the MBO lure and *R*-(+)-limonene + *trans*-conophthorin (LimeCon) and *R*-(+)-limonene + *trans*-conophthorin + *R*-(+)-verbenone (LCV) repellents were placed. (B) indicates placement of repellents for the double dose of *R*-(+)-limonene + *trans*-conophthorin (Dual) in Trial 2 and 3. (C) represents the two 5-m tall poles, each with two sticky traps, one at 2 m and one at 5 m, placed at 5 and 10 m from a subsample of 20 trees (5 trees/treatment) in Trial 2. (D) represents the 3-m tall poles used with a single sticky trap placed 5 m from a subsample of 40 trees (10 trees/treatment) during Trial 3. All semiochemicals (A and B) were placed on the north facing side of trees and poles were randomly assigned to the east or west side of the selected trees (the pole at 10 m was always placed on the opposite side from the 5 m pole in trial two). Sticky traps at 2 m on the trees were randomly assigned to a cardinal direction on the tree and the second sticky trap placed at 4 m on the opposite side of the tree.

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